RAPID SCREENING OF PNH RED CELL POPULATIONS USING THE GEL TEST

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Abstract. Paroxysmal nocturnal hemoglobinuria (PNH), an acquired clonal hematopoietic stem cell defect is underdiagnosed because of its atypical symptoms in some patients and because available methods, which are time consuming and complicated, are not widely used. The purpose of this study is to compare the results of the detection of PNH red cell populations using the PNH gel test and the Ham test. Fifty-eight blood samples obtained from 35 patients and 23 healthy blood donors were tested for PNH by the PNH gel test and the Ham test. It was found that 7 (20%) of the patients were positive for PNH by both tests. Twenty-three blood samples from healthy donors were all negative for PNH by both tests. The overall sensitivity and specificity of the gel test were 100%. This study showed that the PNH gel test was simple and could replace the Ham test as a screening test for PNH. This test would be especially easy to introduce in laboratories that are already using this system for blood grouping and antibody detection.

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell defect. The defect is characterized by the unusual susceptibility of abnormal red blood cell (RBC) populations to the hemolytic action of complement, due to a decrease or lack of surface proteins, most notably CD55 (decay accelerating factor, DAF) and CD 59 (membrane inhibitor of reactive lysis, MIRL). DAF and MIRL are two important glycosyl phosphatidyllinositol-anchored surface proteins, which inhibit complement mediated lysis (Schubert *et al*, 1994; Hillmen *et al*, 1995; Rosse, 1995). The diagnosis of PNH is initially based on the detection of increased red cell susceptibility to lysis, the Ham test or the sucrose lysis test (Beutler, 1995). Routinely, the lytic tests are not widely used because the methods are complicated and time consuming. Presently, the flow cytometry and the sephacryl PNH gel test have also been applied in the investigation and diagnosis of PNH. Both tests make a direct assessment of the known red cell abnormality of PNH, especially the deficiency of DAF and MIRL. Although flow cytometry is increasingly used to differentiate PNH cells in a quantitative procedure, a quick, accurate and simple screening test is needed to replace the lytic tests. Regarding other hematological disorders, the sensitivity of the gel test allows the identification of PNH-affected cells even in small populations (Schubert *et al*, 1991; Pakdeesuwan *et al*, 1997; Meletis *et al*, 1997; Zupanska *et al*, 2002).

In Thailand, the gel test has been used to detect red cell antigen-antibody reactions since its introduction in 1993 (Nathalang *et al*, 1993a,b). Several studies have shown the advantages of its sensitivity and specificity in immunohematological tests (Lapierre *et al*, 1990; Bromilow, 1993; Malyska *et al*, 1994; Nathalang *et al*, 1997). Until now, in order to diagnose PNH, the Ham test is stilled used routinely in hematology laboratory because the flow cytometry is more expensive and not always available. The purpose of this study is to evaluate the PNH gel test as a screening procedure for PNH red cell populations.

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MATERIALS AND METHODS

Subjects

Patients attending the Hematology Unit, Department of Medicine, Phramongkutklao Hospital and the Department of Pediatrics, Ramathibodi Hospital, Bangkok, Thailand were enrolled in the study. There were 4 patients already diagnosed as having PNH, 9 patients with myelodysplastic syndrome (MDS) and 22 patients with acquired aplastic anemia (AA). They comprised 12 females and 23 males with ages ranging from 6 to 74 years and a mean age of 21.5 years. Twenty-three healthy blood donors from the Blood Bank of the Army Institute of Pathology, were also included in this study. They comprised 5 females and 18 males with ages ranging from 23 to 54 years and a mean age of 37.7 years. To increase the validity and reliability of the evaluation, the laboratory technicians were blinded for the diagnosis of the patients.

The PNH gel test

EDTA blood was used for the detection of CD55 (DAF) and CD59 (MIRL) red cell populations. Testing was performed within 24 hours following venepuncture. Fifty microliters of a 1% RBC suspension in a low ionic strength buffer (ID-diluent 2) was added to the three appropriate microtubes of the ID-cards for the PNH test consisting of MIRL, DAF and negative control (PNH-ctl). Then, 50 µl each of anti-MIRL, anti-DAF and negative control were added to the corresponding microtubes. The cards were incubated at 37°C for 15 minutes, centrifuged for 10 minutes in the ID-centrifuge and the reactions were read and interpreted.

Positive reactions of 3+ to 4+ (agglutinated cells forming a red line on the surface of the gel) indicate a normal red cell population with the presence of the corresponding DAF and MIRL antigens. This indicates a negative result for PNH and the PNH-ctl microtube must show a negative reaction (a compact button of cells on the bottom of the microtube). Negative reactions indicate the absence of the corresponding DAF and MIRL antigens. Cells lacking CD55 or CD59 do not agglutinate but form pellets at the bottom of the microtube. This indicates a positive result for PNH. Moreover, double cell populations indicate that part of the cells miss the corresponding antigens CD55 and CD59 and give weak positive PNH test results, as indicated by the manufacturer.

The Ham test

The Ham test was performed in parallel with the gel test for all subjects. Briefly, 10 ml of defibrinated blood was obtained from the patients and those healthy donors with blood groups similar to the patients. After clotting, the serum was separated and the RBCs were washed 3 times with a 0.9% normal saline solution (NSS) and were diluted to a 50% cell suspension in NSS. Then, 500 µl of the patient's serum was added in tubes no. 1, 2, 6 and 7. At the same time, 500 µl of the donor's serum was added into tubes no. 3, 4 and 5. After that, donor serum in tube no. 3 was incubated in a 56°C waterbath for 30 minutes to inactivate complement activity. Fifty microliters of 0.2N HCl was added into tubes no. 2, 3, 5 and 7. Fifty microliters of the 50% RBC suspension of the patients was added into tubes no. 1, 2, 3, 4 and 5 and 50 µl of the donor RBC suspension was added to tubes 6 and 7. All tubes were mixed and then incubated in a 37°C waterbath for 1 hour. Finally, the cell suspension was centrifuged at 1,000*g* for 2 minutes and the hemolysis reactions were read in each tube. Regarding positive reactions for PNH, hemolysis was found only in tubes no. 2 and 5, which contained patient RBCs, acid and fresh serum. Concerning negative reactions for PNH, no hemolysis was found in all tubes (Beutler, 1995).

Statistical methods

The sensitivity and specificity of the PNH gel test were calculated (Ingelfinger *et al*, 1994).

RESULTS

Seven samples of 35 patients (20%) were positive for PNH by the Ham test and the PNH gel test. Three patients were diagnosed as having PNH, one patient with MDS, two patients had acquired aplastic anemia and one patient was diagnosed as combined acquired aplastic anemia with PNH. Double cell populations (positive reactions) were demonstrated by the PNH gel test

Table 1
Patients with positive results for PNH red cells in the PNH gel test; in comparison with the Ham test.

No.	Diagnosis	PNH gel test		Ham test
		Anti-MIRL	Anti-DAF	
1	PNH	3+/-	3+/-	Positive (weak hemolysis)
2	PNH	3+/-	2+/-	Positive (weak hemolysis)
3	PNH	3+/-	2+/-	Positive (weak hemolysis)
4	MDS	3+/-	2+/-	Positive (hemolysis)
5	AA	3+/-	2+/-	Positive (weak hemolysis)
6	AA	2+/-	2+/-	Positive (hemolysis)
7	AA/PNH	3+/-	2+/-	Positive (weak hemolysis)

+/- = Double cell populations, PNH = Paroxysmal nocturnal hemoglobinuria,
MDS = Myelodysplastic syndrome, AA = Aplastic anemia.

in all 7 blood samples. Comparatively, weak to complete hemolysis was found by the Ham test (Table 1). One patient who was diagnosed as MDS with PNH was found to be negative for PNH by both tests. Additionally, 23 blood samples from healthy donors were all negative for PNH by both tests. When the results obtained by both tests were compared, it was found that both the overall sensitivity and specificity of the gel test were 100%, as shown in Table 2.

Table 2
Comparison of the PNH gel test and Ham test in the detection of PNH red cells in 35 patients and 23 healthy blood donors.

PNH gel test	Ham test		Total
	Positive	Negative	
Positive	7	0	7
Negative	0	51	51
Total	7	51	58

Sensitivity = 100%, Specificity = 100%

DISCUSSION

PNH is an acquired stem cell disorder of a clonal nature, resulting in intravascular hemolysis, cytopenia of variable degrees and recurrent thrombotic events. Furthermore, PNH has been described in patients already affected by bone marrow aplasia and conversely, in PNH patients who later develop bone marrow aplasia and rarely, leukemia (Schubert *et al*, 1994; Hillmen *et al*, 1995). Commonly, PNH is underdiagnosed due to its heterogeneity and atypical symptoms that do not resemble classic cases of PNH. Moreover, the available laboratory methods of Ham test to detect PNH are time-consuming and complicated. The use of the hemagglutinating gel test for the diagnosis of PNH seems to open new possibilities (Meletis *et al*, 1997; Zupanska *et al*, 2002). Our results show that the PNH gel test is useful as a screening test for the diagnosis of PNH and probably can replace the Ham test. When the PNH gel test was compared with the Ham test, both positive and negative results were similar. Additionally, one patient formerly diagnosed as MDS with PNH was found to be negative by both tests. This may be due to the varying proportion of normal RBCs which occurs in this patient as a feature of this disease. This is consistent with previous observations (Meletis *et al*, 1997; Zupanska *et al*, 2002). The advantages of the gel test compared to the lytic tests, are that it is simple to use and the results are ready within one-half hour. Also, the gel test allows us to define which proteins (CD59 and/or CD55) are lacking from the patient's defective cells, the same as the flow cytometry test. This is not possible using the lytic tests. Although flow cytometry can diagnose a small clone of defective RBCs and can detect PNH defects on granulocytes in transfused pa-

tients, this technique is not commonly used because it requires more expensive reagents, more complex equipment and better trained personnel (Schubert *et al*, 1991; Pakdeesuwan *et al*, 1997).

In conclusion, the gel test appears to be useful as a screening test for PNH because of its simplicity and increased ability to diagnose PNH. To further validate the usefulness of the PNH gel test, more research with a larger sample size is needed. This test would be especially easy to introduce in laboratories that are already using this system for blood grouping and antibody detection.

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